UTILIZATION OF EXOGENOUS GLUCOSE BY THE REDIAE OF PARORCHIS ACANTHUS (DIGENEA: PHILOPHTHALMIDAE) AND CRYPTOCOTYLE LINGUA (DIGENEA: HETEROPHYIDAE) [1]

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Although the carbohydrate metabolism of some adult parasitic flatworms has been intensively studied (von Brand, 1966; Read, 1961, 1967), the parasitic larval digenetic trematodes have been almost completely neglected. Except for some histochemical observations dealing mainly with the distribution of glycogen, no qualitative or quantitative observations on carbohydrate reserves or carbohydrate metabolism of sporocysts and rediae have been made (Cheng, 1963a; Smyth, 1966). This study was initiated to obtain such information for the rediae of *Parorchis acanthus* (Nicoll, 1906) and *Cryptocotyle lingua* (Creplin, 1825), parasites of marine gastropods.

Materials and Methods

Rediae of *P. acanthus* were recovered from *Thais lapillus* and *Urosalpinx cinerea* (see Stunkard and Cable, 1932; Cable and Martin, 1935), and *C. lingua* rediae from *Littorina littorea* (Stunkard, 1930). Naturally-infected snails were collected near Woods Hole by the Marine Biological Laboratory Supply Department and the authors, and maintained in large tanks with a continuous flow of sea water. Cracked *Mytilus* sp. were provided as food for *T. lapillus* and *U. cinerea*, and the *L. littorea* fed on the algal growth on the sides of the tanks. Snails that were used in these experiments were kept in the laboratory for no more than six days although no statistically significant differences were noted in the carbohydrate content of rediae from snails held for as long as two weeks. *Cryptocotyle* rediae from several snails were always pooled for analysis but when possible, *Parorchis* rediae from individual snails were used.

Infected snail hepatopancreas was teased apart in MBL-formula sea water (Cavanaugh, 1964), following the recommendations of Lockwood (1961) that sea water constituted the best experimental medium for tissues from marine animals. Control experiments indicated that rediae remain alive and active in this solution for long periods and that no measurable carbohydrate leakage occurred in two hours. Free rediae were collected with a capillary pipette and washed with three

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changes of sea water. About 200 *P. acanthus* or 500 *C. lingua* rediae were counted into each experimental vessel. Samples were held exactly 30 minutes in an ice bath; excess fluid was drawn off, and 5 ml. of the appropriate medium were added. Snail hepatopancreas experiments utilized tissue slices of about 20 mg. wet weight that were blotted on hard filter paper, weighed on a torsion balance, and placed in 5 ml. of the appropriate medium for incubation. The time interval between each step was held to a minimum.

The media for the various experimental incubations were: (a) MBL-formula sea water (MEDIUM I); (b) MBL sea water containing 0.1, 0.5 or 1.0 m*M* glucose and an isotope (Glucose-U-C^{14}, New England Nuclear) concentration of 0.1 or 0.25 μc./ml. (MEDIUM II); (c) MBL sea water without $NaHCO_3$ and buffered with 10 m*M* tris(hydroxymethyl)aminomethane-maleate plus 0.5 m*M* glucose and 0.25 μc./ml. of isotope (MEDIUM III). In some experiments, phlorizin (Mann Research Laboratories) was added to MEDIUM II at a final concentration of 5×10^{-4} *M*. The gas phases were atmospheric air, 100% N_2, 2% CO_2–98% air, and 2% CO_2–98% N_2.

Incubations were carried out at 20° C. in a controlled temperature water bath for two hours with constant shaking. Experiments in air were done in open 20-ml. beakers, those in the various controlled atmospheres in screw-cap test tubes in which the medium was equilibrated in a manner similar to that described by Dixon (1966).

Following incubation, the tissues were quickly washed onto a polyvinylchloride membrane (Gelman VM-1) in a vacuum filter funnel and flushed with a large volume of sea water. The filter and adhering tissues were placed immediately into a measured volume of 70% ethanol and extracted for at least 12 hours. Aliquots of the alcoholic extract were used for determinations of radioactivity and chemical analysis of "free" carbohydrates.

The alcohol-extracted tissues were washed with 3 changes of 70% ethanol and then digested with 1 N NaOH at 37° C. The amount of protein was determined in diluted aliquots of the NaOH hydrolysate. The remainder of the sample was heated in a boiling water bath for 30 minutes. Alkali-stable polysaccharide was determined in aliquots from this fraction, or alkali-stable, ethanol-precipitable polysaccharide was precipitated with 1.2 volumes of 95% ethanol. The precipitate was washed several times with 70% ethanol containing 0.1% LiCl, and taken up in a measured volume of distilled water. Aliquots were used for determination of radioactivity and analysis of carbohydrate.

Tissue wet weight and initial carbohydrate were determined on rediae held in sea water in an ice bath for 30 minutes. Dry weight was taken on alcohol-extracted tissues which were dried in an oven at 100° C. for 12 hours. Hemolymph was obtained by heart puncture, and blood from several snails was pooled for analysis.

Carbohydrate was determined by the method of Dubois *et al.* (1956). Glucose was specifically identified by the glucose oxidase method with "Glucostat" (Worthington Biochemical Corporation). Reducing compounds were determined by the Nelson method (Nelson, 1944). Protein was measured by the Folin method (Lowry *et al.*, 1951). For determination of radioactivity, 0.1-ml. portions were plated and counted essentially as described by Simmons, Read and Rothman (1960).

Larger experimental samples would have been desirable, but it was not possible

to remove more rediae from hosts and separate them into appropriately washed and counted groups in an acceptable time period. The amount of labeled carbon incorporated into 200 to 500 rediae was small, but a series of control experiments confirmed the reliability of the data. These showed that: (1) no significant amount of carbon[14] was trapped in the filter, (2) negligible amounts of radioactivity remained in the third wash (2 ml. each) from alcohol-extracted tissues, and (3) rediae held for 5 minutes at 60° C. (heat-killed) did not incorporate radiocarbon during a subsequent two-hour incubation.

All results reported are the average values obtained from at least three replicate experiments unless otherwise stated.

Results

Carbohydrate reserves

The physical, chemical and enzymatic determinations made on the trematode and gastropod tissues and fluids are summarized in Table I. Both the rediae and their snail hosts contained relatively large quantities of polysaccharide and glucose plus at least one other freely-extractable, alkali-resistant and non-reducing sugar. The rediae of *C. lingua* were not analyzed in comparable detail because of their small size, but their carbohydrate content per milligram of protein was very similar to that of *P. acanthus* rediae (Tables I, II, III). Rediae of *P. acanthus* recovered from another gastropod, *Urosalpinx cinerea*, were analyzed, and the amounts of extractable carbohydrate (134 mμMoles/mg. protein) and polysaccharide (1034 mμMoles/mg. protein) were not significantly different from rediae from *T. lapillus* (Tables I, II).

Table I

Physical and chemical determinations

Material	Wet weight (mg.)	Dry weight (mg.)	Prot. (mg.)	NaOH-stable polysaccharide (μMoles)		ETOH-extractable (μMoles)				
						Carbohydrate			Reduc. comp'nds.	
				Non-ppt.	ETOH-ppt.	Total	NaOH boiled	Gluc.	Total	NaOH boiled
Rediae										
P. acanthus[a]	111.5	19.6	15.3	12.86	8.30	1.94	0.59	0.75	3.51	0
C. lingua[b]	—	—	1.0	0.81	—	0.09	—	—	—	—
Hepatopancreas										
T. lapillus	21.8	7.5	4.3	3.44	1.79	—	—	—	—	—
L. littorea	29.3	5.8	2.6	8.57	6.85	—	—	—	—	—
Hemolymph[c]										
T. lapillus	—	—	—	—	—	11.57	9.77	1.79	0.41	0
L. littorea	—	—	—	—	—	12.26	7.47	4.20	0.99	0

[a] About 800 rediae.
[b] About 500 rediae.
[c] Values are μMoles/ml.

TABLE II

Uptake and incorporation of glucose-C^{14} into rediae of Parorchis acanthus. Values are means ± standard deviation.

Medium	Gas phase	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
Initial I	—	21	143 ± 46	—	1126 ± 260	—
(pH 7.8) II	Air	4	93 ± 8	—	1360 ± 220	—
(pH 7.8) II	Air	10	98 ± 17	8 ± 0.4	1382 ± 146	8 ± 1
(pH 7.8) II	N_2	4	89 ± 15	5 ± 0.5	1296 ± 214	2 ± 0.8
(pH 6.6) II	CO_2/N_2	6	121 ± 46	4 ± 1	1235 ± 126	2 ± 1
(pH 6.6) II	CO_2/Air	3	96 ± 11	0.5 ± 0.4	1172 ± 169	2 ± 0
(pH 6.6) III	Air	3	96 ± 6	0.8 ± 0.3	1029 ± 205	5 ± 1
(pH 7.8) III	Air	3	99 ± 12	1 + 0	1124 ± 77	6 ± 1
(pH 7.8) III	N_2	3	90 ± 3	5 ± 0.5	892 ± 63	1 ± 0.6

[a] Expressed as mμMoles glucose/mg. protein.
[b] Radioactivity as mμMoles glucose/mg. protein/2 hrs.
[c] Radioactivity as mμMoles glucose/μMole glycogen/2 hrs.

Glucose utilization

The amount of carbohydrate from rediae before (*i.e.*, initial values) and after incubation in various media under different gas phases is shown in Tables II and III. The variation in the initial values and in those obtained after incubation in glucose-free medium was great enough to obscure any differences among the experimental samples. In incubations of two hours the true rate of absorption of glucose-

TABLE III

Uptake and incorporation of glucose-C^{14} into rediae of Cryptocotyle lingua. Values are means ± standard deviation.

Medium	Gas phase	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
Initial I	—	10	121 ± 49	—	696 ± 193	—
(pH 7.8) II	Air	3	76 ± 5	—	1280 ± 87	—
(pH 7.8) II	Air	5	90 ± 29	38 ± 1	1095 ± 75	18 ± 6
(pH 7.8) II	N_2	3	109 ± 36	32 ± 4	1430 ± 232	trace
(pH 6.8)	CO_2/N_2	3	106 ± 24	20 ± 5	1349 ± 79	trace

[a,b,c] Symbols as in Table II.

C^{14} from the medium is obscured by molecular exchange and metabolism. However, significant differences in incorporation of glucose-C^{14} into polysaccharide under the various experimental conditions were noted.

The rate of incorporation in *P. acanthus* rediae incubated under an atmosphere of N_2 or CO_2/N_2 was about one-fourth the rate of those incubated in air (Table II). That part of this depression of incorporation was due to the lowered levels of oxygen seems likely, but it appears that carbon dioxide also adversely affected the incorporation rate. This is suggested by similar low rates of incorporation in rediae incubated under either CO_2/N_2 or CO_2/air mixtures (Table II). It is difficult to separate the biological effects of carbon dioxide in solution and the resultant change in pH but it appears unlikely that the lowered pH resulting from gassing with carbon dioxide had any marked effect since the incorporation rate of rediae incubated in air at a similar pH (6.6) was only slightly depressed (Table II). The small change in rate observed may have been due to the absence of sodium bicarbonate or to the presence of the tris-maleate buffer in the medium (Medium III) because the incorporation rate of rediae incubated in this medium at pH 7.8 was slightly lower than that of rediae incubated in MBL sea water (Medium II) (Table II).

The results of experiments with rediae of *C. lingua* paralleled those of *P. acanthus*. The rate at which glucose-C^{14} was incorporated into polysaccharide in air was about twice that of *P. acanthus*, and the rate was significantly reduced in atmospheres lacking oxygen and containing carbon dioxide (Table III).

The apparent uptake and incorporation rates of glucose-C^{14} in *P. acanthus* and *C. lingua* rediae were lower in media containing a low concentration of the glycoside, phlorizin (Table IV).

The initial and post-incubation level of carbohydrate and the rate of uptake and incorporation of glucose-C^{14} into polysaccharide by slices of the hepatopancreas of *T. lapillus* and *L. littorea* were determined also. Values were more variable than those obtained for the rediae but incorporation by the hepatopancreas under the different experimental conditions paralleled that of the parasites (Table V). The rate of incorporation in *T. lapillus* tissue more nearly approximated that in *P. acanthus* rediae, while the rate in *L. littorea* more nearly approximated that in *C. lingua* rediae (Tables II, III, V).

TABLE IV

The effect of phlorizin (5×10^{-4} M) on the uptake of glucose-C^{14} into rediae. Values are means ± standard deviation where number of experiments permit.

Species	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
P. acanthus					
Control	3	104 ± 74	7 ± 0	1303 ± 212	8 ± 3
Phlorizin	3	104 ± 11	1 ± 0	1224 ± 84	6 ± 0
C. lingua					
Control	2	53	5	1576	13
Phlorizin	3	76 ± 2	3 ± 0	1304 ± 109	9 ± 2

[a,b,c] Symbols as in Table II.

TABLE V

Uptake and incorporation of glucose-C^{14} into hepatopancreas tissue slices. Values are means ± standard deviation where number of experiments permit.

Species	Medium	Gas phase	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
T. lapillus	Init.	—	3	199 ± 85	—	469 ± 240	—
	I	Air	3	148 ± 13	—	446 ± 54	—
	II	Air	3	172 ± 35	7 ± 4	678 ± 383	6 ± 2
	II	CO_2/N_2	3	123 ± 23	4 ± 2	363 ± 141	3 ± 0.6
	II	N_2	3	147 ± 30	6 ± 1	463 ± 107	4 ± 1
L. littorea	Init.	—	2	501	—	527	—
	I	Air	2	380	—	338	—
	II	Air	2	366	3	491	20
	II	CO_2/N_2	2	389	1	521	4
	II	N_2	2	407	1	492	4

[a,b,c] Symbols as in Table II.

DISCUSSION

The information on carbohydrate metabolism of trematodes has been subjected to extensive review recently and will not be recounted in any detail here (see von Brand, 1966; Cheng, 1963a; Read, 1961, 1967; Smyth, 1966).

Adult trematodes have a pronounced carbohydrate metabolism, but comparatively little is known about the biochemistry of larval stages. Larval trematodes characteristically contain a substantial carbohydrate reserve which apparently is important in their energy metabolism (von Brand, 1966; Cheng, 1963a), but the meager data available suggest differences in the ability of the various larval stages to utilize this reserve. Thus the miracidium of *Fasciola hepatica* can utilize exogenous substrates and labeled glucose is incorporated into glycolytic intermediates (Bryant and Williams, 1962). *Schistosoma mansoni* cercariae do not develop and emerge from infected snail tissues *in vitro* if adequate quantities of glucose and trehalose are not present in the culture medium (Chernin, 1964). *Cotylurus brevis* cercariae live longer in water to which glucose has been added but they are unable to resynthesize glycogen from glucose (Ginetsinskaya and Dobrovolski, 1963). Certain amino acids prolong the survival of *Fascioloides magna* rediae *in vitro* (Friedl, 1961a, 1961b) and increase the respiration rate of rediae of *Himasthla quissetensis* (Vernberg and Hunter, 1963) but glucose and several other common sugars are without effect in either worm.

The rediae of *P. acanthus* and *C. lingua* contain relatively large quantities of tissue sugars including glucose and are able to take up exogenous glucose and incorporate significant amounts into polysaccharide *in vitro*. Their carbohydrate reserves and glucose utilization rates are comparable to those of some adult parasitic flatworms (see von Brand, 1966).

The importance of carbohydrate in the metabolic activities of mollusks has been well documented (see Martin, 1961; Awapara and Simpson, 1967). It is sufficient to reiterate that the range of blood sugar levels is quite broad both within and between species and that glycogen levels fluctuate under various conditions, *e.g.*, seasonal, nutritional, reproductive. We found *T. lapillus* and *L. littorea* contained

considerable amounts of polysaccharide, glucose and other freely-extractable sugars (Table I). In our experiments, the hepatopancreas of each snail absorbed and incorporated glucose at a rate similar to that of the rediae from that host, and incubations under the various gas phases gave parallel results (Tables II, III, V). The similarities are significant if parasite and host tissue are in competition for available glucose.

A considerable portion of the alcohol-soluble carbohydrate recovered from the rediae and from the snails' hemolymph was not glucose and on further analysis was shown to be alkali-stable and non-reducing (Table I). This component may be the disaccharide trehalose, which is widely distributed among invertebrates. Fairbairn (1958) reported this sugar in 71 species representing the major invertebrate phyla, including an adult trematode, *F. hepatica*, and the gastropods, *T. lapillus* and *L. littorea*. Further, the only other free sugar he found was glucose.

In the present experiments, gassing with nitrogen or a carbon dioxide-nitrogen mixture may not have produced absolute anaerobiosis but the oxygen tension must have been reduced to an extremely low level. The marked depression in uptake and incorporation under these oxygen-deficient atmospheres strongly suggests that oxygen is of considerable importance in the metabolism of these rediae and their hosts. There are no data available on the oxygen tensions within the tissues and circulatory systems of the gastropod hosts, but Vernberg (1963) suggests that it is low and variable. All stages in the life cycle of digenetic trematodes utilize oxygen when it is available, but the respiration rate of *H. quissetensis* rediae showed less dependency on reduced oxygen tensions than did any of the other stages of that species (Vernberg, *loc. cit.*).

Our results indicate that carbon dioxide had an inhibitory effect on glucose incorporation that was not attributable to the absence of oxygen nor to the fall in pH produced by carbon dioxide in solution (Table II). In a related study, carbon dioxide stimulated the incorporation of radioglucose into polysaccharide by *C. lingua* adults recovered from gulls (McDaniel, unpublished observations).

It is possible that the adverse effects of oxygen lack and the presence of unlabeled carbon dioxide on radioglucose incorporation in rediae may be related to carbon dioxide fixation mechanisms.

Because of fluxes and metabolism, the values for uptake of glucose-C^{14} from the medium obtained in two-hour incubations are not measures of initial entry rates. However, phlorizin, a glycoside known to be an inhibitor of mediated glucose uptake (Crane, 1960), inhibited the entry of glucose into the rediae (Table IV). It seems possible, therefore, that mediated processes may also be operative in rediae.

Rediae have a simple sac-like gut with a muscular pharynx anteriorly and are able to ingest host cells, but probably there is absorption through the body wall. There is a correlation between phosphatase activity and absorptive function in tissue distribution [although little more can be said about their relationship (see Crane, 1960; Read, 1966)] and alkaline phosphatase activity has been demonstrated in the tegument and in the cells lining the gut of *Echinoparyphium* sp. rediae (Cheng, 1964). The fine structure of the surface of the rediae of *P. acanthus* (Rees, 1966), *C. lingua* (P. Krupa, personal communication) and *F. hepatica* (K. E. Dixon and E. H. Mercer, unpublished observations) is strongly suggestive of an absorptive surface. Mitochondria are numerous in these teguments and "microvilli" increase the surface area available for absorption.

The gut is relatively small in a fully grown redia but with its muscular pharynx does function in the mechanical damage of host tissues (Cheng and Snyder, 1962; Wright, 1966) and frequently contains partly-digested material (Rees, 1966; Cheng, 1963b; Cheng and James, 1960; Krupa, personal communication; Dixon and Mercer, unpublished observations). However, no secretory cells have been found associated with the gut of rediae except for some unicellular glands opening into the esophagus of *P. acanthus* (Rees, 1966). The enzymes responsible for the digestion of the host's tissues may come from the host's cells ruptured mechanically during ingestion.

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Summary

1. The rediae of *P. acanthus* and *C. lingua* and their gastropod hosts, *T. lapillus* and *L. littorea*, contained substantial amounts of "free" carbohydrate and polysaccharide. The free fraction consisted of glucose and at least one other sugar, probably trehalose.

2. Rediae and host heptopancreas tissues absorbed exogenous glucose and incorporated significant amounts into polysaccharide *in vitro*.

3. Glucose absorption in rediae was markedly depressed by a low concentration of phlorizin, an inhibitor of mediated transport systems. Incorporation into polysaccharide was greater in air than under nitrogen, and atmospheres containing carbon dioxide were inhibitory independent of the presence of oxygen.

4. The results support the hypothesis that rediae absorb nutrients through the body surface in addition to ingestion of particulate matter into the gut. The rediae have substantial amounts of glucose available to them *in vivo* and the potential to absorb and utilize glucose has been demonstrated. Since the apparent rates of glucose utilization by parasite and host tissues are similar, the rediae are probably not at a disadvantage in the competition for carbohydrate.

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